



Molecular Cloning and Allergenicity of Pen j 1, a Major Allergen of Kuruma Prawn, *Penaeus japonicus*

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Tropomyosins have been identified as a common allergen in crustaceans, but their allergenicity is not well understood. In the present study, we isolated an allergen, Pen j 1, a tropomyosin from kuruma prawn *Penaeus japonicus*, and determined its N-terminal amino acid sequence. The cDNA encoding the allergen was cloned by 5'- and 3'-rapid amplification of cDNA ends (RACE), and was found to code for a protein which consists of 284 amino acid residues. Sequencing analyses indicated for the first time that mature tropomyosin is formed by the elimination of a leader peptide of nine amino acid residues. To elucidate the binding sites of IgE antibodies in the sera of shrimp-sensitive patients, various recombinant peptides were expressed in *Escherichia coli* as fusion proteins with glutathione *S*-transferase (GST), and the examined with regard to reactivity with IgE antibodies. The IgE-binding epitopes were found to locate over the whole sequence of the allergen, and the IgE antibodies in the sera were found to recognize strongly its C-terminal region.

Key words: shrimp allergy; *Penaeus japonicus* tropomyosin; cDNA cloning; recombinant protein; IgE-binding epitope

The prevalence of food allergy is a serious problem throughout the world. In Japan, recently the Welfare and Labor Ministry examined foodstuffs that cause the disorder in people ingesting them using persons selected by random sampling and reported that shrimp and crab was the most frequent foodstuff of the ones examined.¹⁾ Subsequently, the Ministry investigated allergenic foodstuffs with reference to patients visiting hospitals for treatment of their food allergic symptoms, and found that allergy often occurred due to the consumption of egg, cow's milk, wheat, buckwheat, shrimp, and peanut,

in decreasing order.²⁾ The difference between the two reports can be explained by idea that people, especially adults exhibiting the disorder by ingestion of shrimp or crab would avoid consuming them and thus do not visit the hospital. This means that shrimp and crab, as well as egg, cow's milk, and wheat, are noteworthy allergenic foodstuffs in Japan, and that it is very important to clarify their allergenicity for the prevention of and recovery from food allergic symptoms.

Crustaceans are frequent causes of allergic reactions to seafoods throughout the world.^{3–6)} Since most edible crustaceans belong to the order Decapoda (including shrimps or prawns), previous studies for crustacean allergens have been performed with decapods. The only major allergen in decapods has been identified as tropomyosin, a 37-kDa protein constituting thin myofilaments together with actin and troponin. It bound to approximately 75% of IgE antibodies in sera from shrimp-sensitive individuals.^{7–9)} In addition, it has been reported that tropomyosin is a common allergen in other crustaceans, such as lobsters *Panulirus stimpsoni* (Pan s 1)¹⁰⁾ and *Homarus americanus* (Hom a 1),¹¹⁾ crab *Charyabdis feriatius* (Cha f 1),¹²⁾ mollusks such as squid *Todareus pacificus* (Tod p 1),¹³⁾ and oyster *Crassostrea gigas* (Cra g 1).¹⁴⁾

Crustacean tropomyosins exhibit IgE cross-reactivity with one another and also with those from various other invertebrates, including house-dust mites and cockroaches.^{15–21)} Elucidation of their amino acid sequences is the most important task to understand IgE cross-reactivity among tropomyosins of different species. As for crustacean tropomyosins,^{9–12,22)} those from phylogenetically unrelated vertebrate,^{23–25)} also have been examined with regard to their amino acid sequences by the cDNA cloning technique. These studies indicate that amino acid sequence identity is very high among

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Abbreviations: GST, glutathione *S*-transferase; IPTG, isopropyl- β -thio-D-galactopyranoside; LB, Luria-Bertani; PVDF, polyvinylidene fluoride; RACE, rapid amplification of cDNA ends

tropomyosins from invertebrates, but not between invertebrates and vertebrates, suggesting that IgE-binding epitopes are common among allergenic invertebrate tropomyosins. Hence studies on tropomyosin as a common allergen in crustaceans have been performed in Western countries.

Although patients in Japan suffering from allergic responses to shrimp or crab have increased in number due to increased consumption, as described above, no systematic research on the allergenicity of shrimp has been carried out. In the present paper, we describe the isolation, characterization, and molecular cloning of Pen j 1, a major allergen from kuruma prawn, popularly consumed in Japan. The expression and the IgE-binding activity of various recombinant peptides of the allergen in *Escherichia coli* (*E. coli*) are also reported.

Materials and Methods

Sera of patients sensitive to shrimp. Sera of shrimp-sensitive patients were obtained from Kumamoto University Hospital. Informed consent was obtained. The experiments were carried out in accordance with the ethical standards formulated in the Helsinki Declaration. All subjects were suffering from allergic reactions such as urticaria, asthma, diarrhea, and anaphylaxis within 1 h following the ingestion of shrimp. Sera from 17 patients with shrimp allergy (aged 1–47 years, total IgE, 0.13–6.01 µg/ml) were obtained and stored at –80 °C (Table 1). Clinical manifestations of shrimp allergy showed that 12 of the 17 patients had skin reactions, including urticaria (6/17), angioedema, (4/17) and pruritus (2/17). Several patients had gastrointestinal (4/17) and respiratory (4/17) symptoms. Food-dependent exercise-induced anaphylaxis occurred in only one patient (No. 8).

Determination of total IgE antibodies and Pen j 1-specific IgE antibodies in the patients' sera. Fifty microliters of anti-human IgE antibody (Chemicon International, Temecula, CA, 1 µg/ml) were added to the wells of a 96-well microplate and incubated at 4 °C overnight. The wells were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS/T) at 37 °C for 1 h, then washed 3 times with PBS/T. The wells were incubated with precisely diluted patients' sera at 37 °C for 1 h and washed as described above. The human IgE binding to 1st antibody on the wells was further reacted with biotinylated anti-human IgE

antibody (American Qualex Antibodies, San Clemente, CA) and then with horseradish peroxidase-conjugated streptavidin (Roche Diagnostics, Indianapolis, IN). The immunocomplexes on the wells were detected as described previously.²⁶⁾ The amount of IgE antibody in the samples was determined with a calibration curve obtained with human IgE, myeloma (Calbiochem, Darmstadt, Germany) as a standard specimen. To determine Pen j 1-specific IgE antibody, purified Pen j 1 was coated on a 96-well microplate instead of the above-mentioned anti-human IgE antibody.

Purification of tropomyosin from kuruma prawn. Pen j 1 was purified from live kuruma prawn, *Penaeus japonicus*, which was purchased locally according a modification of the method of Shanti *et al.*⁸⁾ Briefly, 15 g of sliced prawn were homogenized in 30 ml of cold 50 mM KCl/2 mM NaHCO₃ buffer (pH 8.0) containing 10 mM 2-mercaptoethanol (2-ME), and soluble sarcoplasmic proteins were removed by centrifugation at 4 °C for 20 min at 10,000 × g. The precipitate obtained was resuspended with the same buffer and homogenized, and washing of the precipitate was repeated twice. An equal volume of 99.5% ethanol was added to the precipitate, and the suspension was filtered through a cheese cloth. The residue was washed twice with 100% ethanol and then with diethyl ether. The residue was allowed to dry at room temperature overnight in a draft chamber. Finally, about 1.5 g of muscle protein powder was obtained. The proteins in the dried powder were extracted at 4 °C overnight with 30 ml of 20 mM Tris–HCl (pH 7.5) containing 1 M KCl and 10 mM 2-ME. After centrifugation at 10,000 × g for 20 min at 4 °C, the supernatant was adjusted to pH 4.5 with 1 M HCl and allowed to stand for 1 h at room temperature. After centrifugation, the precipitate formed was dissolved in 30 ml of 1 mM NaHCO₃, and the solution was adjusted pH 7.5 with 1 M KOH and allowed to stand for 1 h at room temperature. After clarification by centrifugation, 2-ME and solid KCl were added to the supernatant at final concentrations of 10 mM and 1 M respectively. Then isoelectrical precipitation was done twice more. Finally, the proteins in the supernatant obtained were fractionated with solid ammonium sulfate. The precipitate formed between 40–60% ammonium sulfate was collected by centrifugation, dissolved in 1 mM NaHCO₃, and dialyzed overnight at 4 °C against the same solution. The protein concentration was determined with a Protein Assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard protein.

Analysis of the N-terminal amino acid sequence of the purified Pen j 1. The purified Pen j 1 was separated on a gel by SDS–PAGE and electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilion P, Millipore, Billerica, MA). The

Table 1. Demographic, Serologic, and Clinical Characterization of the Subjects

The subjects were characterized by age, sex, and clinical diagnosis of allergies. IgE reactivity for the whole protein of kuruma prawn and purified Pen j 1 was analyzed by immunoblot assay and ELISA.

Patient number	Age	Sex	Allergenic food	Symptom	Total IgE	Pen j 1-IgE
					(µg/ml)	
1	1	M	Egg (shrimp, crab, squid)	Cough, wheeze	6.01	1.11
2	1	F	Wheat (shrimp, fish)	Urticaria	0.56	N.D.
3	2	M	Egg, milk, shrimp	Vomiting	3.01	0.01
4	2	M	Egg, milk, shrimp, wheat	Angioedema, wheeze	2.09	N.D.
5	5	M	Shrimp, crab, fish, banana, latex	Urticaria	4.88	N.D.
6	6	F	Shrimp	Urticaria, dyspnea	0.63	N.D.
7	7	F	Egg, milk, shrimp	Urticaria, dyspnea	0.95	N.D.
8	16	M	Shrimp	Exercise-induced anaphylaxis	7.63	0.16
9	18	M	Shrimp	Wheeze, dyspnea, pruritus of pharynx	1.11	0.04
10	20	M	Shrimp	Pruritus of pharynx	1.02	0.14
11	23	M	Shrimp	Angioedema, dyspnea	1.5	0.02
12	25	M	Shrimp	Abdominal pain	1.08	0.01
13	29	M	Egg, shrimp, crab	Urticaria	1.43	N.D.
14	39	M	Shrimp	Angioedema	1.11	N.D.
15	40	M	Shrimp	Urticaria, vomiting, abdominal pain	2.33	0.25
16	43	M	Shrimp	Vomiting	0.25	N.D.
17	47	M	Shrimp	Angioedema, dyspnea	0.13	N.D.

N.D., not detected.

band corresponding to Pen j 1, stained with CBB R-250, was cut off and subjected to the N-terminal amino acid sequence analysis with 473 protein sequencer (Applied Biosystem).

SDS-PAGE and immunoblotting. Protein samples were analyzed by SDS-PAGE on polyacrylamide gels under reducing conditions by the method of Laemmli.²⁷⁾ For immunostaining, the separated proteins on the gels were electrotransferred onto nitrocellulose membranes. The membrane strips were incubated overnight at 4°C with patients' sera (diluted 1:5), and the bound IgE antibodies were reacted with biotin-labeled anti-human IgE antibody (diluted 1:1,000, Seikagaku Kogyo, Tokyo), and then with horseradish peroxidase-labeled streptavidin (diluted 1:5,000, Roche Pharmaceuticals, Nutley, NJ). The immuno-complexes on the membrane were detected in the same manner as described in a previous paper.²⁸⁾ On the other hand, the epitope mapping with patients' sera using the recombinant peptides was examined in the same manner as described above, except that ¹²⁵I-labeled anti-human IgE (GE Healthcare, Little Chalfont, Bucks) was used as the second antibody, instead of biotin-labeled anti-human IgE antibody, and the immunocomplexes on the membrane were detected as described previously.²⁹⁾

Isolation of poly(A)⁺RNA and first-strand cDNA synthesis. Total RNA was prepared from live specimens of kuruma prawn (*Penaeus japonicus*). The prawn samples were ground to powder in a glass mortar filled with liquid nitrogen, homogenized in 10 vol. of Isogen (Nippon Gene, Tokyo), and mixed vigorously with 2 vol. of chloroform. The aqueous solution obtained by centrifugation at 4°C for 15 min at 10,000 × *g* was mixed with an equal volume of isopropanol, and this was centrifuged after standing for 10 min, as described above. Crude RNA in a pellet was dissolved in a small volume of DEPC-treated water, precipitated in the presence of 0.25 M NaCl with 2 vol. of 99.5% ethanol, and stored at -80°C. The pellet, containing total RNAs formed, was washed with cold 80% ethanol, again dissolved in a small volume of DEPC-treated water, and used to prepare poly(A)⁺RNA using a mRNA Isolation kit (Roche Diagnostics, Indianapolis, IN).

3'-Rapid amplification of cDNA ends (RACE) and 5'-RACE-ready first-strand cDNAs were synthesized from 1.0 µg of poly(A)⁺RNA using a SMART[™] RACE cDNA amplification kit (Clontech, Mountain View, CA) following the user's manual.

Isolation of cDNA encoding Pen j 1. The primers used in this study were designed from published nucleotide sequences of crustacean tropomyosin cDNAs. P1 (5'-CAGGCGATGAAGCTGGAGAAGGATA-3'), a sense primer for QAMKLEKDA, the sequence corresponding to the 9–16 amino acid sequence, and P2 (5'-CAGAGCGGCC-ACCTCGCCCTCAGC-3'), an antisense primer for AEGEVAAL, the sequence corresponding to the 81–88 amino acid sequence, were prepared as gene-specific primers for 3'-RACE and 5'-RACE cloning, respectively. First, 3'-RACE to obtain a 3'cDNA sequence using a gene-specific primer, P1, and UPM included in the kit was performed under the following conditions: initial denaturation, 5 min at 95°C; amplification, 1 min at 95°C, 1 min at 70°C, and 3 min at 72°C, 36 cycles; final elongation, 10 min at 72°C. Two PCR products, with about 1,400 bp and 1,250 bp, were amplified, separated on an analytical 8% polyacrylamide gel, and extracted from the gel. PCR amplification was done as described above using two DNA fragments isolated as templates with the nested primers, NP1 (AACGCCATGGATAGGGCGGATAC-3') and NUP, in the kit. When a DNA fragment of 1,400 bp was used in PCR, a specific cDNA was amplified. The 1,400-bp DNA fragment was separated by electrophoresis on a 1% low-melting agarose gel, and the gel pieces possessing fragments were cut off. The DNA fragments in the gel pieces were purified with a QIAEX II Gel Extraction kit (Qiagen, Valencia, CA), subcloned into the pGEM-T Easy vector included in the TA cloning kit (Promega, Madison, WI), and sequenced. Next, 5'-RACE to obtain a 5' cDNA sequence using the P2 primer and the UPM primer which includes a part of the sequence of SMART II oligo, was performed in the same manner as described above, and many PCR products were obtained. To detect a specific DNA fragment among them, Southern blot analysis was performed using the above-mentioned 1,400-bp DNA fragment labeled with an AlkPhos Direct Labeling and Detection kit (GE

Healthcare, Little Chalfont, Bucks). Since an approximately 400-bp DNA fragment exhibited a positive signal, DNA fragments formed by PCR with 5'-RACE were separated by electrophoresis on an 8% polyacrylamide gel, and a gel piece possessing the 400-bp fragment was cut off. The DNA fragment in the gel piece was extracted with TA buffer at room temperature overnight, subcloned, and sequenced as described above.

DNA sequencing. Sequence analysis was performed using a SequiTherm[™] Long-Read[™] Cycle Sequencing kit (Epicentre Technologies, Madison, WI), IRD800 Infrared Dye Labeled primer M13 Forward/Reverse (Nisshinbo Research Center, Tokyo), and an LI-COR DNA sequencer model 4000L (LI-COR, Lincoln, NE). Both strands of six different clones were analyzed to establish the final sequence of the amplified fragment.

Expression of recombinant Pen j 1 in E. coli. The designations and nucleotide sequences of the primers used in the construction of an expression system for the full-length (F10) and the partial peptides (F1–F9) of Pen j 1 are summarized in Fig. 5 and Table 2. The above-mentioned 3'-RACE ready first-strand cDNA was amplified by PCR using primers with the BamH I and EcoR I sites, in the 5'- and 3'-regions respectively. The PCR products were subcloned into pGEM-T Easy vector and sequenced in the same manner as described above. After the sequence was confirmed, the inserted cDNA fragments were digested with BamH I and EcoR I and ligated to the respective sites of pGEX-6P-1 vector (GE Healthcare, Little Chalfont, Bucks) for expression of the glutathione S-transferase (GST) fusion protein. To express the fusion protein in *E. coli*, the recombinant plasmid (pGEX/F1–F10) was transformed in *E. coli* BL21 and cultured in a Luria-Bertani (LB) plate at 37°C. Transformation into *E. coli* BL21, screening for recombinant Pen j 1 peptide-producing clones, and intracellular expression were performed by the method described previously.³⁰⁾

Purification of recombinant Pen j 1 peptides F1–F10. BL21 cells transformed with pGEX/F1–F10 were grown at 37°C in LB medium containing ampicillin with shaking overnight, and then a 50-ml aliquot was regrown in 1.0 liter of new LB medium. After 3 h, isopropyl-β-thio-D-galactopyranoside (IPTG, final 0.1 mM) was added in the culture medium, and the culture of BL21 was continued for 7 h at 37°C. Unless otherwise stated, all subsequent operations were done at 4°C. The medium was centrifuged at 8,000 × *g* for 5 min, and the pellet obtained was suspended in 100 ml of 10 mM sodium phosphate buffer (pH 7.3) containing 0.15 M NaCl, 2 mM ME, and 1 mM EDTA. The cells were broken by 2 min of sonication, and the lysate was centrifuged at 20,000 × *g* for 20 min. The supernatant was applied to a glutathione-Sepharose 4B column (2 ml) equilibrated with the binding buffer, and the column was washed with 20 ml of the same buffer. The GST-fusion protein was eluted with 10 mM reduced glutathione in 50 mM Tris/HCl, pH 8.0. Fractions containing recombinant Pen j 1 as a GST-fusion protein were pooled and dialyzed against PBS (pH 7.4), and subjected to SDS-PAGE analysis and immunoblotting.

Results

Purification and sequence analysis of the Pen j 1 protein

Pen j 1 was purified from a live kuruma prawn (15 g), as described in "Materials and Methods." The crude proteins were extracted from the muscle protein powder prepared, and the Pen j 1 in the extract was purified by repetitive precipitation at an isoelectric point of pH 4.5 and fractionation with 40–60% ammonium sulfate. The protein obtained was analyzed by SDS-PAGE, and the allergen gave a single band the corresponding to a protein of 34 kDa molecular mass on the gel (Fig. 1). Finally, 23.2 mg of Pen j 1 was obtained from the prawn (15 g). After SDS-PAGE and electroblotting on a nitrocellulose membrane, the Pen j 1 on the membrane

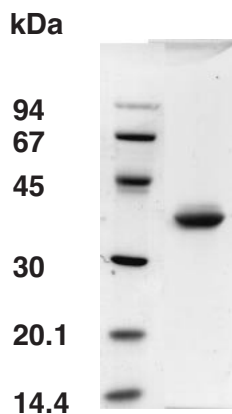


Fig. 1. SDS-PAGE of Purified Tropomyosin (Pen j 1) from Kuruma Prawn.

The protein was electrotransferred on a PVDF membrane after SDS-PAGE separation and subjected to analysis of the N-terminal amino acid sequence.

was examined with regard to its glycan moiety using the PAS test. The protein was found to be negative on this test (data not shown), indicating that no glycan moiety was linked with the protein.

The N-terminal amino acid sequence of Pen j 1 electroblotted on a PVDF membrane was determined by Edman degradation, and the first 20 N-terminal amino acid residues were AMKLEKDNAMDRA DT-LEQQN. The sequence was found to be perfectly identical with the deduced amino acid sequences (10A–29N) of crustacean tropomyosins reported previously.

Binding of the protein in the kuruma prawn extracts and the purified tropomyosin with IgE antibodies in patients' sera

The IgE reactivities of all the sera were examined by immunoblotting with whole proteins in kuruma prawn and by sandwich ELISA against the purified tropomyosin, Pen j 1 (Table 1). Fifty percent of the patients' sera exhibited binding potency with tropomyosin and contained significant Pen j 1-specific IgE antibodies (0.02–1 µg/ml). The IgEs in the sera of three patients (No. 9, No. 10 and No. 15) reacted to only one tropomyosin of whole proteins from kuruma prawn (Fig. 2), indicating that Pen j 1 is also a major allergen. Moreover, we observed indirect histamine release under Pen j 1 stimulation of healthy human basophils (total IgE, 40 ng/ml) sensitized with IgE antibodies in the serum of patient No. 15 (data not shown).

cDNA cloning and sequencing

Since identities among the amino acid sequences of crustacean tropomyosins are extremely high, based on the nucleotide sequences of the cDNA encoding them, three oligonucleotides (P1, NP1, and P2) were synthesized and used as primers in the amplification of cDNA fragments encoding the allergen. A cDNA fragment of 1,340 bp including a poly (A)⁺-tail was amplified by PCR with 3'-RACE ready first-strand cDNA as the template and primers P1 and UPM. On the other hand, when 5'-RACE ready first-strand cDNA as the template was amplified by PCR with primers P2 and UPM, a

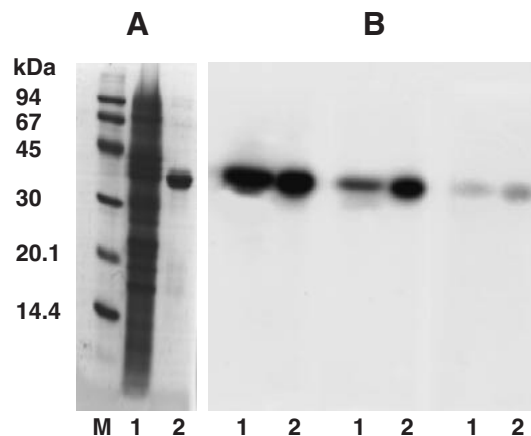


Fig. 2. Immunoblots of Whole Protein from Kuruma Prawn and Purified Pen j 1 with the Sera of Shrimp-Sensitive Patients.

A kuruma prawn was homogenized with 5 vol. of buffer, and 0.5-ml aliquots of the homogenate were heated with equal volume of sample buffer for SDS-PAGE at 100 °C for 10 min. The supernatant (lane 1) obtained by centrifugation of the reaction mixture at 10,000 × g for 20 min and the purified Pen j 1 (lane 2) were applied to a 15% polyacrylamide gel and analyzed. The proteins separated on the gel were electrotransferred onto a nitrocellulose membrane and stained with Ponceau S (panel A). The other membrane was cut into three strips for incubation with sera of shrimp-sensitive patients, followed by ¹²⁵I-labeled anti-human IgE antibody testing. The IgE-bound proteins on the membrane were detected by autoradiography (panel B), as described in the text. M, standard protein markers.

cDNA fragment of 366 bp as a specific product was obtained. These DNA fragments were subcloned into pGEM-T Easy vector and sequenced, as described in "Materials and Methods." The sequence data for these recombinant plasmids indicated that two cDNA fragments, of 1,340 bp and 366 bp, included an overlapping sequence. By combination of the two sets of sequence data obtained from the recombinant plasmids, a 1,462-bp cDNA was established (Fig. 3). As shown in Fig. 3, the ATG codon, encompassing nucleotide positions 96–98, was most likely to be the translation initiation codon referring to the sequence of brown shrimp (*Penaeus aztecus*) tropomyosin, Pen a 1.⁸⁾ The complete coding sequence included a 852-bp open reading frame between positions 96 and 947, encompassing 284 amino acid residues. The overall sequence of the deduced amino acid of the cDNA cloned was very similar to that of other crustacean tropomyosins (Fig. 4). Although the N-terminal amino acid sequence of the isolated Pen j 1 was found to be included in the deduced amino acid sequence, it was located at amino acid sequences 10–29, indicating that the cDNA codes for a polypeptide of 284 amino acids including a leader peptide of nine residues.

Expression of recombinant Pen j 1 peptides (F1–F10) in E. coli

In order to characterize the IgE-binding epitopes on Pen j 1, we investigated the expression of recombinant Pen j 1 peptides F1–F10 (Fig. 5, Table 2). Plasmids, pGEX/F1–F10, were constructed to express the recombinant peptides as GST fusion proteins in *E. coli* under the control of the *lac* promoter. *E. coli* BL21 cells transformed with these plasmids were cultured as described above. When *E. coli* BL21 harboring the expression vectors were cultured in the absence of IPTG, no fusion proteins (>26 kDa) of the peptide

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1. GAGTCGAGAGTACCTCTCCCGGTGAGACTCTCCACTCCGAGCTTTCTCAGTTCTCTTTTGGTTGAGCACCTCTCAACA
AAACCGCGCCATGAGCGCCATCAAGAAGAAGATGACGGCGATGAAGCTGGAGAAGGATAAGCCCATGGATAGGCGGATACCC
P1 NP1
M D A I K K K M Q A M K L E K D N A M D R A D T L
TTGAACAGCAGAACAGGAGGCCAACACAGGGCTGAGAAGAGCGAGGAGGTTACAACTTCAGAAAGGATGACAGAAC
E Q Q N K E A N N R A E K S E E E V H N L Q K R M Q Q L
TTGAGAACGACCTTGACAGGTGACGAATCTTGTGAAGGCTAACATCCAGCTTGTGAGAGGACAGGCGCTCTCTAAGC
E N D L D Q V Q E S L L K A N I Q L V E K D K A L S N A
CTGAGGCGAGGTGGCGCTCTGAACCGCGCATCCAGCTTCTGAGGAGGACCTGGAGCGCTCTGAGGAGCGCTCAACACCG
F2
E G E V A A L N R R I Q L L E E D L E R S E E R L N T A
CCACCACCAAGCTGGCGAGGCTCCAGGCGCGAGCGAGTCCGAGCGCATGCGCAAGGTGCTCGAGAACGCGTCCCTGTCCG
T T K L A E A S Q A A D E S E R M R K V L E N R S L S D
ACGAGGAGCGCATGAGCGCTCGAGAACGAGTCAAGGAGGCGCGATTCCTGGCTGAGGAGCGACAGGAAATACGACGAGG
E E R M D A L E N Q L K E A R F L A E E A D R K Y D E V
TTGCCGTAGCTGGCATGGTTGAGGCTGACCTTGAGCGTGTGAGGAGCGTCCGAGAGCTGGTGAATCAAGATCGTCGAGC
A R K L A M V E A D L E R A E E R A E T G E S K I V E L
TTGAGAGGAGCTGCGTGTGCTGGCAACACCTGAAGTCCCTGAGGTGTCTGAGGAGAGGCTAACACGCGCGAGGAGGCT
E E E L R V V G N N L K S L E V S E E K A N Q R E E A Y
ACAAGGAGCAGATTAGACCTCTACCAACAGCTTAAGGCGGTGAGGCGGTGCTGAGTTCGCGAGAGGTCTGTGCGAGAGC
K E Q I K T L T N K L K A A E A R A E F A E R S V G K C L
TCCAGAAGGAGTTCGACGCTCGAAGACGAATGGTTAACGAAAGGAGAAGTACAACTCCATACCGACGAGCTGGACCGA
Q K E V D R L E D E L V N E K E K Y K S I T D E L D Q T
CTTTCAGGAACTGTCTGGCTACTAAACACTCTCTGCTCCAACTGTTCTGCCATCTCTCTCTATGCCCTCAGCTGGCTGT
F S E L S G Y ***
AACCTATTTTTCATAAGCTTATGTCATTAGTTTATAAGAACTTTTACTTTTCTCTTTTATGTGTTCATTTGACCTT
CGATTGAGTCAATATCATCAATAATCATTCGATAACGTTGGTGAAGTCTGCCATATAGTAAATTTTAAATGTTATTAAGGTA
ATCTTTTATTCAGAACCTAGATTTTGAAGTTCATTTCAAAAGGACAGGTTTCATGTATTTCTATGGCTTAATAACATAAGAAAT
AAATATTTTATTCATAATGAATTTGTTTGTGTTTACGCTGTTATCCGACGACGACCATGACCAACGATGAAGCGAGTGGGAC
TAANATGAGCCTAGGACGCTGCTGCTGCCNCGTCAGCGGTGGACTGCTGTAAATATCTCAACCATCTGTTCACAGATGAA
CTTGTATGAAAAA
1462

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Fig. 3. Nucleotide Sequence of cDNA Encoding Pen j 1 and the Deduced Amino Acid Sequence.

The deduced amino acid sequence is shown under the nucleotide sequence of the cDNA. The double underlining under the amino acid sequence represents the amino acid residues of N-terminus of the purified Pen j 1. The three arrows refer to primers P1, NP1, and P2, used in PCR. Asterisks show the stop codon of the cDNA.

	1		R1	75
Pen j 1	MDAIAKKKQAMKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Pen a 1	MDAIAKKKQAMKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Met e 1	-----MKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Pan s 1	-----MKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Por s 1	MDAIAKKKQAMKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Der p 10	MDAIAKKKQAMKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Per a 7	MDAIAKKKQAMKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Chicken	MDAIAKKKQAMKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
Rabbit	MDAIAKKKQAMKLEKDNAMDRADTLEQQNK	EANNRAEKSEEEVHN	LQKRMQQL	ENDLDQVQESLLKANIQLVEKD
	76		R2	150
Pen j 1	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Pen a 1	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Met e 1	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Pan s 1	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Por s 1	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Der p 10	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Per a 7	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Chicken	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
Rabbit	KALSNAEAGEVAALNRRIQLLEEDLERSEER	LNTATTKLAESAQA	ADESERMRKVLEN	RSLSDEERMDALENLKE
	151		R4	225
Pen j 1	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Pen a 1	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Met e 1	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Pan s 1	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Por s 1	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Der p 10	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Per a 7	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Chicken	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
Rabbit	ARFLAEAEADRKYEVARKLAMVEADLERAE	ERAETGESKIVLE	EEELRVVGN	NLKSLEVSEEKANQREAYKEQI
	226		R5	284
Pen j 1	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Pen a 1	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Met e 1	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Pan s 1	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Por s 1	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Der p 10	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Per a 7	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Chicken	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY
Rabbit	KTLTNKLKAAEAEAEFAERSVQKLQKEVD	RLEDELVNEKEKY	SITDELDTF	SELSGY

Fig. 4. Amino Acid Sequence Alignment of Allergic and Non-Allergic Tropomyosins.

Accession numbers (GenBank data): *Paninus aztecus* (Pen a 1), DQ151457; *Metapenaeus ensis* (Met e 1), U08008; *Panulirus stimpsoni* (Pan s 1), AF030063; *Portunus sanguinolentus* (Por s 1), EF143836; *Dermatophagoides pteronyssinus* (Der p 10), Y14906; *Periplaneta americana* (Per a 7), AF106961; chicken tropomyosin, NM205401; rabbit α -tropomyosin, NM001105688. The code (R1–R5) represents the location of IgE-binding epitopes on Pen a 1 as reported by Ayuso *et al.*⁽³⁹⁾

fragments with GST (26 kDa) were detected in the total cell extracts by SDS–PAGE followed by staining with Ponceau S. On the other hand, when *E. coli* were cultured in the presence of 0.1 mM IPTG, the recombinant peptides which were predicted to correspond to the GST fusion proteins were newly observed on the gel by SDS–PAGE, indicating that the recombinant peptides (F1–F10) were expressed in *E. coli* (data not shown).

All of the GST fusion proteins were estimated to constitute about 50% of the total protein in the *E. coli*

cells by the above-mentioned SDS–PAGE analysis (Fig. 6, lane 1). As shown in Fig. 6, a large portion of the recombinant peptides (GST-F7) occurred in soluble fraction of the cell lysates (Fig. 6, lane 2). Hence we purified it from crude extracts of *E. coli* lysate by affinity chromatography with a glutathione-Sepharose 4B column (Fig. 6, lane 3). Thus, the purified peptides were subjected to SDS–PAGE analysis, followed by immunoblotting with the sera of shrimp-sensitive patients.

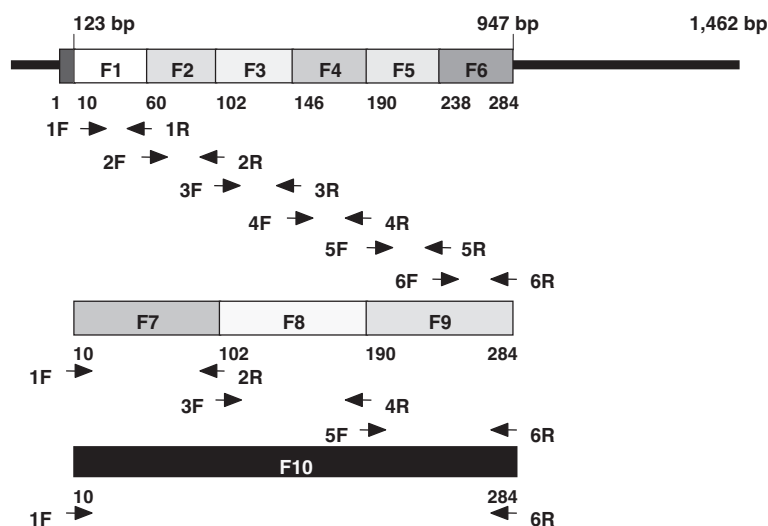


Fig. 5. Designation of the Primers Used to Obtain the cDNA Fragments Encoding the Peptide Fragments of Natural Pen j 1.

Table 2. Designations and Nucleotide Sequences of the Primers Used for Recombinant Peptides of Pen j 1 (An overview for the synthesis of recombinant peptides is shown in Fig. 5)

Primer	Corresponding amino acid sequence			Primer sequence (5'-3')
Sense				
1F	10	AMKLEKD	16	AGCTATGGATCCGAGATGAAGCTGGAGAAGGA
2F	60	VQESLLK	66	GACCTTGGATCCGTGCAGGAATCCTTGCTGAA
3F	102	SEERLNT	108	GACCTTGGATCCCTCTGAGGAGCGCCTCAACAC
4F	146	NQLKEAR	152	TCAAGTGGATCCAACCAGCTCAAGGAGGCCCG
5F	190	IVELEEE	196	CTTGAAGGATCCATCGTCGAGCTTGAGGAGGA
6F	238	RAEFAER	244	TAGGCTGGATCCCGTGCTGAGTTCGCCGAGAG
Antisense				
1R	53	LENDLDQ	59	AGGATTGAATTCACTGGTCAAGGTCGTTTCTCAA
2R	95	LEEDLER	101	AGATCTGAATTCAGCGCTCCAGGTCCTCCTCGA
3R	139	ERMDALE	145	CCTTGAGAATTCAGTCCAGGGCGTCCATGCGCT
4R	183	AETGESK	189	CAAGCTGAATTCAGTTTGATTACACAGTCTCGG
5R	231	KLKAAEA	237	CGAACTGAATTCAGGCCTCAGCCGCCTTAAGCT
6R	278	FSELSGY	284	CAGAGAGAATTCAGTAGCCAGACAGTTCGCTGAA

Binding of the recombinant peptides to IgE antibodies in the sera of patients sensitive to shrimp

The above-mentioned purified GST-F10, a recombinant full-length polypeptide of Pen j 1, was immunoblotted with IgE antibodies in the serum of shrimp-sensitive patient. The reactivity was similar to that of natural Pen j 1 (Fig. 7). This indicates that the recombinant protein was correctly folded and was equivalent to its natural counterpart. On the other hand, the natural and recombinant Pen j 1 did not react to human IgE and IgE antibodies in the serum of a mite-sensitive patient used as the control. Thus it was shown that recombinant peptides obtained as GST fusion proteins can be used as probes to identify the IgE-binding regions on the polypeptide chain of Pen j 1.

Hence we investigated the identification of IgE-binding regions on the allergen by immunoblotting analyses using the recombinant peptides (F1–F10) with various molecular masses and the sera of three patients (No. 9, No. 10, and No. 15), who showed IgE-binding reactivity to F10. As shown in Fig. 8, all three subjects strongly recognized the recombinant peptides F7, F8, and F9 which Pen j 1 was divided into three peptides with similar molecular size. For further location of the epitopes recognized by these IgE antibodies, each

peptide was divided into two segments (F1–F6), as shown in Fig. 5, but of these small peptides, only three (F4, F5, F6) were immunoblotted with all the sera, and the IgE-binding reactivity with the peptides were found to be similar one another. In contrast, no IgE-binding signal was observed in two peptides, F1 and F2, even though F7 was recognized by all the sera, suggesting that one or some of the epitopes on F7 bestride the boundary between the C-terminal region of F1 and the N-terminal region of F2. F3 was immunoblotted with only one serum, of patient No. 15. These results indicate that the IgE-binding epitopes of the allergen locate over the whole sequence on the polypeptide chain of Pen j 1 and predominantly at the C-terminal site.

Discussion

Seafoods, including crustacean, are known to provoke gastrointestinal as well as systemic allergic reactions,^{3–6)} and, among them, crustaceans, including shrimps and prawns, are most frequently associated with IgE-mediated type-1 hypersensitive reactions in food-allergic patients.³¹⁾ The only major allergen identified in crustaceans is the muscle protein tropomyosin. It binds approximately 75% of the shrimp-specific IgE

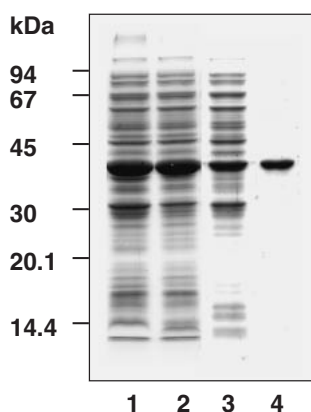


Fig. 6. SDS-PAGE of the Recombinant Peptides of Pen j 1 (GST-F7) Obtained at the Various Purification Steps.

Lane 1, cell lysate by sonication; lane 2, soluble fraction of cell lysate; lanes 3 and 4, flow-through and elution fractions on glutathione-Sepharose 4B column. The other recombinant peptides showed the same pattern as GST-F7 on SDS-PAGE.

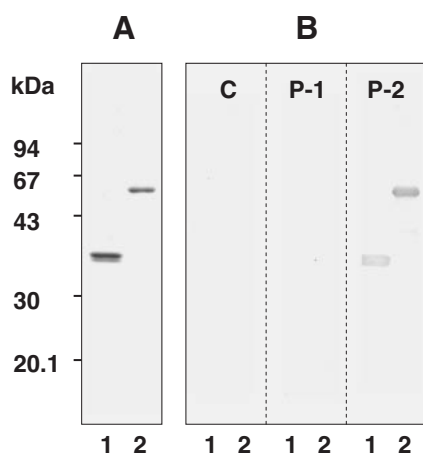


Fig. 7. Immunoblot of Natural Pen j 1 and Recombinant Tropomyosin with Patients' Sera.

Purified Pen j 1 and recombinant tropomyosin (GST-F10) were electrophoresed on SDS-polyacrylamide gel (12.5%), and then electrotransferred to a nitrocellulose membrane. The membrane was divided into several strips. One strip was stained with Ponceau S (A). The other strips (B) were subjected to immunostaining with allergic patients' sera (P-1, house dust mite-sensitive; P-2, shrimp-sensitive patient No. 15). Additionally, a strip was immunostained with standard human IgE as a negative control (C). In this experiment, HRP-conjugated anti-human IgE antibody was used as a second antibody, and the immunocomplex was visualized by peroxidase reaction with 0.3% H_2O_2 and 0.75% 4-chloro-1-naphthol as substrates. Lane 1, purified natural Pen j 1; lane 2, GST-F10.

from shrimp-sensitive patients.⁷⁻⁹) Recently, arginine kinase³²) and sarcoplasmic calcium-binding protein³³) have been identified as new allergens in black tiger prawn, *Penaeus monodon*, and the former also in pacific white shrimp, *Litopenaeus vannamei*.³⁴) About 50% of the sera from the 17 shrimp-allergic patients tested in this study exhibited IgE reactivities with kuruma prawn proteins, in which Pen j 1 predominantly bound to IgE antibodies in the sera. Although the other half patients had few amounts of allergen-specific IgE, they showed allergic symptoms (Table 1). These results suggest that shrimp-allergic reactions are triggered in individuals not only through an IgE-mediated mechanism, but also by non-IgE-related systems. Although food allergy is thought to be induced primarily by an IgE-mediated

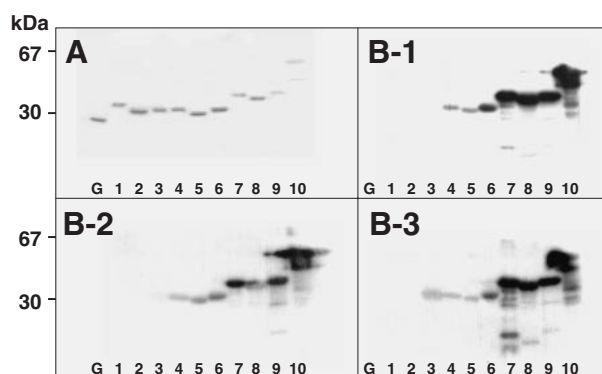


Fig. 8. Immunoblotting of Recombinant Peptides with Patients' Sera.

The purified recombinant peptides (GST-F1 to F10) were electrophoresed on 15% polyacrylamide gel, and then electrotransferred to nitrocellulose membranes. One membrane was stained with Ponceau S (A), and the others (B-1 to B-3) were immunostained with the patients' sera. Detection of the immunocomplexes on the membranes was carried out as described in the legend to Fig. 2. Numbers in the membranes refer to the applied recombinant peptides, GST-F1 to GST-F10. Patients' sera: B-1, patient No. 9; B-2, patient No. 10; B-3, patient No. 15. G, GST.

mechanism, evidence suggests that non-IgE-related mechanisms might also be involved in the pathogenesis of shrimp allergy. Presently food allergy is divided into three groups, named IgE-mediated food allergy (type-I), mixed IgE and non-IgE-mediated, and non-IgE-mediated (cellular) food allergy,^{35,36}) but tropomyosin from kuruma prawn was elucidated to be a major allergen for some patients, and it caused shrimp-induced allergic reactions by an IgE-mediated mechanism (Fig. 2).

In this study, we investigated the molecular characterization of Pen j 1 by protein sequencing and cDNA cloning to clarify the allergenicity of the allergen. Purified Pen j 1 was obtained as a homogeneous 34-kDa polypeptide band on SDS-PAGE (Fig. 2). The 20 N-terminal amino acid residues determined by Edman degradation were AMKLEKDNAMDRTLEQQN. This sequence is entirely consistent with the deduced amino acid sequence (10A–29N) of Pen j 1 (Fig. 3) and with crustacean tropomyosins reported previously. The open reading frame of the Pen j 1 cDNA was found to encode a polypeptide of 284 amino acid residues without Cys, Pro, and Trp (Fig. 3). The first nine amino acids were absent in the mature peptide, as determined by protein sequencing, and they constituted a leader peptide (Fig. 3). Although much information on the primary structure of allergenic and non-allergenic tropomyosins has been gained by the cDNA cloning technique (Fig. 4), the N-terminal amino acid sequences in their mature proteins have never been elucidated. Hence we purified tropomyosins from *Penaeus monodon* and *Metapenaeus joyneri* in the same manner as for Pen j 1 and determined the amino acid residues of their N-termini. The N-terminal amino acid sequences of purified Pen m 1 and Met j 1 were AMKLEKDNA and AMKLEKDNAM respectively, suggesting that the deduced amino acid sequences from other tropomyosin cDNAs also include a leader peptide consisting of nine amino acid residues. Surprisingly, the amino acid sequence of the leader peptide was most highly conserved among muscle tropomyosins not only from invertebrates but also from vertebrates (Fig. 4). Gen-

erally, tropomyosin forms a coiled-coil structure that binds head-to-tail along the helical actin filament.³⁷⁾ The ends of 284-residue tropomyosins are believed to overlap by nine amino acids. Moraczewska and Hitchcock-DeGregori³⁸⁾ investigated the functions of the N- and C-terminal overlap regions using recombinant tropomyosins, in which nine amino acids were truncated from the N-terminal, C-terminal, or both ends of tropomyosin, and stated that end-to-end interactions are not required for high-affinity binding to acto-myosin S1. The relationship between the structure and the function of tropomyosin should be elucidated. The fact that a 275-residue tropomyosin in which nine amino acids were truncated from the N-terminus occurred as a mature protein in nature provides useful information on the structural chemistry of tropomyosin.

We constructed an expression vector, pGEX/F10, to obtain the recombinant protein of mature Pen j 1 and succeeded in overexpressing a fusion protein of 275-residue recombinant polypeptide with GST (GST-F10) in *E. coli*. The purified GST-F10 was found to bind only to IgE antibodies in shrimp-sensitive patients' serum (Fig. 7), indicating that the recombinant peptides obtained as GST fusion proteins can be used as probes to identify the IgE-binding regions on the polypeptide chain of Pen j 1. We tried to identify IgE-binding regions using smaller recombinant peptides with different amino acid sequences (F1–F9), which were synthesized in the same manner F10 was. The results obtained immunoblotting analysis of their recombinant peptides with the sera of shrimp-sensitive patients revealed that the IgE-binding regions of Pen j 1 located broadly over the whole sequence on the polypeptide chain of Pen j 1 and predominantly in the C-terminal region (Fig. 8). Shanti *et al.*⁷⁾ imperfectly determined two IgE-binding regions of Pen i 1, consisting of amino acid residues 50–66 and 153–161, by analyzing IgE-binding capacities of nine tryptic peptides with a patient serum. Ayuso *et al.*³⁹⁾ synthesized 46 overlapping peptides (length, 15 amino acids; offset, 6 amino acids) spanning the whole length of Pen a 1 (284 residues) and systematically analyzed the IgE-binding activities of the peptides with sera from shrimp-allergic subjects. Based on the results, five major IgE-binding regions were identified, as follows: region 1, 43–57; region 2, 85–105; region 3, 133–147; region 4, 187–201; and region 5, 247–284. As shown in Fig. 4, the amino acid sequence identities among the crustacean tropomyosins were extremely high, and 100% identity was observed between Pen a 1 and Pen j 1, tropomyosins from two species of *Penaeus* shrimp (brown shrimp) and prawn (kuruma prawn). Hence it is speculated that the location of the IgE-binding epitopes of Pen j 1 with Japanese patients' sera are similar to those of Pen a 1. In practice, as shown in Fig. 8, the IgE-binding regions of Pen j 1 included regions 3, 4, and 5 identified by Ayuso *et al.*³⁹⁾ and two regions identified by Shanti *et al.*⁷⁾ Further, the IgE-binding potency and frequency to F6 with patients' sera were the highest in the recombinant peptides, F1–F6, suggesting that more epitopes exist on the F6 peptide. Ayuso *et al.*³⁹⁾ also demonstrated that region 5, corresponding to F6, was the particularly important IgE-binding epitope, since the strongest IgE reactivities of shrimp-allergic individuals were directed towards this region.

In conclusion, the present study yielded information on the structural chemistry of Pen j 1 and its IgE-binding epitopes and should facilitate further molecular study to understand crustacean allergy in detail.

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